

A Monoclonal Antibody against α -Smooth Muscle Actin: A New Probe for Smooth Muscle Differentiation

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Abstract. A monoclonal antibody (anti- α sm-1) recognizing exclusively α -smooth muscle actin was selected and characterized after immunization of BALB/c mice with the NH₂-terminal synthetic decapeptide of α -smooth muscle actin coupled to keyhole limpet hemocyanin. Anti- α sm-1 helped in distinguishing smooth muscle cells from fibroblasts in mixed cultures such as rat dermal fibroblasts and chicken embryo fibroblasts. In the aortic media, it recognized a hitherto unknown population of cells negative for α -smooth muscle actin and for desmin. In 5-d-old rats, this population is about half of the medial cells and becomes only $8 \pm 5\%$ in 6-wk-old animals. In cultures of rat aortic media SMCs, there is a progressive increase of this cell population together with a progressive decrease in the number of α -smooth muscle actin-containing stress fibers per cell. Double

immunofluorescent studies carried out with anti- α sm-1 and anti-desmin antibodies in several organs revealed a heterogeneity of stromal cells. Desmin-negative, α -smooth muscle actin-positive cells were found in the rat intestinal muscularis mucosae and in the dermis around hair follicles. Moreover, desmin-positive, α -smooth muscle actin-negative cells were identified in the intestinal submucosa, rat testis interstitium, and uterine stroma. α -Smooth muscle actin was also found in myoepithelial cells of mammary and salivary glands, which are known to express cytokeratins. Finally, α -smooth muscle actin is present in stromal cells of mammary carcinomas, previously considered fibroblastic in nature. Thus, anti- α sm-1 antibody appears to be a powerful probe in the study of smooth muscle differentiation in normal and pathological conditions.

ALTHOUGH actin is one of the most conserved eukaryotic proteins, it is expressed in mammals and birds as six isoforms characterized by two-dimensional (2D)-PAGE and amino acid sequence analysis (14, 49, 56, 57, 59). Four of them represent differentiation markers of muscle tissues and two are found practically in all cells (56, 57). Actin isoforms show >90% overall sequence homology, but only 50–60% homology in their 18 NH₂-terminal residues (56, 57). The NH₂-terminal region of actin appears to be a major antigenic region (2, 5, 34) and may be involved in the interaction of actin with other proteins such as myosin (31).

Little is presently known about the function of actin isoforms. It has been shown that the relative proportions of actin isoforms are different in smooth muscles of different organs (12, 47; Skalli, O., J. Vandekerckhove, and G. Gabbiani, manuscript submitted for publication) and change within the same population of smooth muscle cells (SMCs)¹ during development (23, 24), pathological situations (11, 21), and different culture conditions (35, 46, 50). In nonmuscle cells, a difference in the proportions of β - and γ -cytoplasmic actins has been reported between normal and tumoral T-lympho-

cytes (28). All these studies have been performed on cell populations by means of 2D-PAGE (14, 49, 59) or by analysis of the NH₂-terminal peptides (56, 57) since, with possibly one exception (38), antibodies specific for a single actin isoform are lacking.

We have produced a monoclonal antibody against a synthetic decapeptide having the sequence of the NH₂-terminal peptide of α -smooth muscle actin, which is specific for this isoform. We report here the characterization of this antibody, the identification of an α -smooth muscle actin-negative population among rat and human arterial SMCs and the localization of α -smooth muscle actin in myoepithelial cells of mammary and salivary glands, as well as in stromal cells of mammary carcinomas previously considered fibroblastic in nature.

Materials and Methods

Peptide Synthesis and Antibody Production

The decapeptide was synthesized by classical solution technique (61) using various coupling procedures and a combination of acid-labile and hydrogenolytically cleavable protecting groups. The fully protected polypeptide Ac-Glu(OBut)-Glu(OBut)-Glu(OBut)-Asp(OBut)-Ser(But)-Thr(But)-Ala-

1. *Abbreviation used in this paper:* SMC, smooth muscle cell.

Leu-Val-Cys(Acm)-NH₂ was partially deprotected by trifluoroacetic acid treatment and purified by hydrophobic interaction chromatography on a highly porous styrene-divinylbenzene copolymer. (Ac = acetyl [CH₃CO-]; Acm = acetamidomethyl [CH₃CONH-CH₂-]; But = tert-butyl [ether]; Et = ethyl; OBut = tert-butyl ester). The acetaminomethyl group (Acm) was cleaved by exposure of the peptide to mercury(II)-acetate. The resulting peptide was homogeneous as judged by thin layer chromatography and analytical HPLC and showed the expected amino acid composition upon amino acid analysis of a hydrolyzed sample.

The peptide was coupled to BSA by the thiol function of its cysteine residue via a 3-maleimidopropionate moiety as anchoring group (20). 27 mg BSA was dissolved in 2 ml 0.1 M phosphate buffer, pH 7.5, and reacted with 34 mg *N*-succinimidyl-3-maleimidopropionate (dissolved in 0.3 ml dimethylformamide). The reaction mixture was stirred for 25 min at pH 7.5 before the pH was adjusted to pH 6.0 by addition of 1 N HCl. The excess of reagent was removed by gel filtration on a Sephadex G-25S column, with 0.05 M phosphate buffer, pH 6.0, as eluent. The modified carrier protein was eluted in the first peak. This solution was reacted with 18.5 mg peptide for 20 h and again chromatographed on a Sephadex G-25S, as described above. The peptide-carrier conjugate was concentrated in an Amicon cell and dialyzed against 0.01 M phosphate buffer, pH 7.2, containing 0.145 M NaCl. The degree of substitution was 21 mol of peptide per mol BSA as determined by amino acid analysis (48). The peptide was coupled to keyhole limpet hemocyanin by the same procedure and a degree of substitution of 41.5 mol of peptide per mol of hemocyanin was achieved.

BALB/c mice (4–6 wk old) were immunized by intradermal and subcutaneous injections of 50 µg of the hemocyanin-coupled peptide emulsified with an equal volume of Freund's complete adjuvant (final volume 200 µl). Two boosts of 50 µg of the same peptide conjugate emulsified with an equal volume of Freund's incomplete adjuvant (final volume 200 µl) were administered at 2-wk intervals. 3 d before fusion a mouse was injected intravenously with 50 µg of the conjugate in 100 µl sterile PBS. On the fusion day, the mouse spleen was removed aseptically and teased in DME. The spleen cell suspension was fused with Sp 2/0 myeloma cells according to the protocols of Galfre and Milstein (13) and De St. Groth and Scheidegger (7). Hybridoma cells were plated into five 96-well microtest plates (Falcon Labware, Oxnard, CA) with 10⁴ peritoneal macrophages per well and grown for 2 wk in medium containing hypoxanthine/aminopterin/thymidine (HAT) and 20% fetal calf serum (FCS). Anti-actin-secreting hybridomas were identified by solid-phase ELISA and immunofluorescence (see below). A clone secreting α -smooth muscle actin-specific antibodies (anti- α -asm-1) was obtained after a precloning and two clonings by limiting dilution. The other hybridomas were transferred to a 24-well culture plate (Falcon Labware) and grown to confluence before being frozen in hypoxanthine/thymidine medium containing 20% FCS and 10% DMSO.

ELISAs were performed on plates coated with either platelet (37) or bovine aortic (51) actin. Pure actin (0.5 µg in 50 µl 50 mM bicarbonate buffer, pH 9.6) was added to each well of a polyvinylchloride microtiter plate (96 wells; Dynatech Laboratories, Inc., Alexandria, VA) and allowed to adsorb overnight at 37°C. For screening, plates were rinsed with PBS and incubated for 1 h at 37°C with 40 µl of hybridoma supernatants. After three rinsings with PBS, plates were incubated for 1 h at 37°C with 40 µl of rabbit anti-mouse IgG and IgM or rabbit anti-mouse IgG labeled with horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted at 1:500 in PBS, containing 0.5% BSA and 0.05% Tween 80. After three rinsings in PBS, plates were developed by adding 40 µl of a solution of 5 mM 5-aminosalicylic acid plus 0.005% H₂O₂. Hybridoma supernatants positive for actin were further tested on cryostat sections of chicken gizzard (see below).

Immunofluorescent Staining

Double immunofluorescent staining with anti- α -asm-1 and various other antibodies was performed on 4-µm-thick cryostat sections, freshly isolated cells, and cell cultures as previously described (12, 46). Anti- α -asm-1 was used together with the following affinity-purified polyclonal antibodies: guinea pig anti-vimentin (22), rabbit anti-desmin (22), guinea pig anti-prekeratin (41), and rabbit anti-aortic smooth muscle myosin (3; Benzana, G., O. Skalli, and G. Gabbiani, manuscript in preparation). This last antibody is essentially similar to the anti-smooth muscle myosin antibody previously described by Larson et al. (26). In some instances, rabbit anti-rat Factor VIII antibodies (gift of Dr. G. Elemer, Department of Pathology, University of Lausanne, Lausanne, Switzerland) were also used.

Tissue sections were fixed for 5 min in acetone at -20°C and air dried. Cell cultures and cytopins were fixed for 5 min in methanol at -20°C. Dilution of supernatants was done in PBS containing 3 mM EGTA in order

to prevent the actin-depolymerizing activity present in serum (6). IgG concentration of the supernatant was estimated after purification on protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Anti- α -asm-1 was used constantly at an IgG concentration of 5 µg/ml. Affinity-purified anti-vimentin, anti-desmin, anti-prekeratin, and anti-aortic smooth muscle myosin were used at a final IgG concentration of 0.85, 0.025, 0.20, and 0.02 mg/ml, respectively. As second antibodies, we used TRITC-labeled goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) diluted 1:30, FITC-labeled goat anti-rabbit IgG (Behringwerke, Marburg-Lahn, Federal Republic of Germany), and FITC-labeled goat anti-guinea pig IgG diluted 1:20 (Cappel Laboratories). Cell counts were done independently by two researchers on 300 to 500 cells. Results are expressed as mean \pm SEM.

Photographs were taken with a Zeiss photomicroscope equipped with epillumination, using plan achromate $\times 40/1.0$ or $\times 63/1.4$ objectives, on 3M 640T color slide films (3M Co., Savona, Italy) or HP5 Ilford black and white film (Ilford Co., Basel, Switzerland).

Immunoblotting Experiments

Antibody specificity was determined by immunoblotting of monodimensional gels of whole tissue homogenates or purified actins and 2D-PAGE of actin isoforms. For SDS PAGE, tissue samples were prepared as described previously (23) and electrophoresed on a 5–20% gradient gel (25). 2D-PAGE separation of actin isoforms was done by isoelectric focusing using pH 4–6.5 ampholines (Pharmacia) followed by migration on a 10% SDS PAGE (32). To determine the actin isoforms recognized by anti- α -asm-1, gels were transferred to nitrocellulose papers (52) which were incubated with anti- α -asm-1 at an IgG concentration of 5 µg/ml in Tris-buffered saline containing 3% BSA and 0.1% Triton X-100, for 2 h at room temperature. After three rinsings in Tris-buffered saline, a second incubation was done with rabbit anti-mouse IgG labeled with horseradish peroxidase (Nordic Immunological Laboratories) diluted 1:1,000 in Tris-buffered saline containing 3% BSA and 0.1% Triton X-100 for 1 h at room temperature.

We also controlled that actin isoforms did transfer equally well to the nitrocellulose paper with a rabbit actin antibody recognizing all isoforms by ELISA. The nitrocellulose paper was first incubated with this antibody at a dilution of 1:10 and then with a goat anti-rabbit IgG labeled with horseradish peroxidase (Nordic Immunological Laboratories) at a dilution of 1:500. Peroxidase activity was always revealed with 10 mM Tris-HCl, pH 7.4, containing 0.025% diaminidase and 0.0075% H₂O₂.

Tissue Culture

SMCs were isolated from the thoracic aortic media of 5-d-old or 6-wk-old rats and from human thoracic aortic media by enzymatic digestion (21, 23, 46). They were then cytocentrifuged at 125 g for 5 min with a Shandon cytocentrifuge (Shandon Scientific Co., Ltd, London) and processed for immunofluorescence. SMC cultures were prepared from the thoracic aorta of 6-wk-old female Wistar rats by enzymatic digestion with collagenase and elastase as previously described (19) with minor modifications (46). Immunofluorescence was also done on primary and passaged aortic SMCs grown on glass coverslips (46).

Rat fibroblasts were isolated from rat dermis after enzymatic digestion for 2 h at 37°C with DME containing 4 mg/ml collagenase (*C. Histolyticum*; Type I, 290 U/mg) (Sigma Chemical Co., St. Louis, MO), 0.5 mg/ml elastase (Pancreatopeptidase; Type III, 95 U/mg) (Sigma Chemical Co.), and 1 mg/ml soybean trypsin inhibitor (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany). They were grown in DME containing 10% FCS. Multipassaged cells were plated on glass coverslips at a density of 3×10^4 cells/ml for 1 or 2 d before immunofluorescent staining.

We examined also the following cell lines grown in DME containing 10% FCS and plated on glass coverslips: human embryo lung fibroblasts (gift of Dr. B. Azzarone, Institut National de la Santé et de la Recherche Médicale, Villejuif, France), chick embryo fibroblasts (gift of Dr. C. Meric, Department of Molecular Biology, University of Geneva, Geneva, Switzerland), and BC3H1 cells (American Type Culture Collection, Rockville, MD), a cell line derived from vascular SMCs of a mouse brain tumor (50).

Immunofluorescent Staining with Anti- α -asm-1 of Primary SMCs in the G1 or S-G2 Phase of the Cell Cycle

According to autoradiographic experiments, the first DNA synthesis during primary culture of rat aortic SMCs takes place between 24 and 48 h after

seeding (46). Hence, by cytofluorometry 36 h after plating, we separated SMCs entering for the first time in the S or G2 phase of the cell cycle from SMCs that remained in G1. Cells were labeled with the DNA binding dye Hoechst 33342 (gift of Mr. P. Zaech, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), and G1 and S-G2 populations were sorted on the basis of their fluorescent intensity with an EPICS V flow cytometer (Coulter Electronics Inc., Hialeah, FL), using an argon ion laser (Spectra-Physics Inc., Mountain View, CA) at 350-nm excitation wavelength as previously described (46). Aliquots of 2×10^4 sorted cells in the G1 or S-G2 phases were immediately air dried on a glass slide, fixed, and processed for immunofluorescence as described above.

Results

Antibody Characterization

Rabbit immunization with hemocyanin-coupled α -smooth muscle NH₂-terminal decapeptide resulted consistently in high titer antibodies staining SMCs, fibroblasts, and various epithelial cells, but not myocardial and striated muscle cells (data not shown).

Hybridoma supernatants from fusions between Sp2/0 myeloma cells and lymphocytes from a mouse immunized with the α -smooth muscle NH₂-terminal peptide conjugate were first screened by ELISA against aortic or platelet actin. Eighty-one of 480 supernatants gave a positive reaction with one or both actins; 55% of them were positive only for aortic actin and did also react on plates coated with the peptide used for immunization, but coupled to BSA.

Previous work (47; Skalli, O., J. Vandekerckhove, and G. Gabbiani, manuscript submitted for publication) has shown that all mammalian smooth muscle tissues contain α -smooth muscle actin. To test the cross-reactivity of anti- α sm-1 with γ -smooth muscle actin, we selected chicken gizzard which contain mainly γ -smooth muscle actin with some β -non-muscle and γ -nonmuscle actins but not α -smooth muscle actin (24, 39, 55). The primary sequence of γ -smooth muscle actin from chicken gizzard and from mammalian smooth muscle have been shown to be identical (55). About 80% of the aortic actin-positive supernatants stained vascular but not parenchymal SMCs in cryostat sections of chicken gizzard. We cloned cells from one of these fusion wells, obtaining a clone secreting anti- α sm-1, whose specificity was further tested by immunoblotting and immunofluorescence. Anti- α sm-1 was of the IgG class as determined by ELISA and immunofluorescence.

Immunoblotting on SDS PAGE of total extracts from different tissues showed that the antibody reacts with aortic actin but not with actin from fibroblasts (β - and γ -cytoplasmic), striated muscle (α -sarcomeric), and myocardium (α -myocardial) (Fig. 1 *a*). Similar results were obtained when chicken gizzard, platelets, or striated muscle were blotted after being loaded in amounts ~ 10 -fold greater than aortic actin, using IgG concentrations ranging from 50 μ g/ml to 0.1 μ g/ml (Fig. 1 *b*). ELISAs gave similar results. Anti- α sm-1 specificity was also assayed on blots of actin isoforms separated by 2D-PAGE. We used human myometrium (Fig. 2, *a-c*) which contains a slight predominance of α -smooth muscle actin, and human gastric muscle (Fig. 2, *d-f*) which contains an excess of γ -smooth muscle over α -smooth muscle actin (Skalli, O., J. Vandekerckhove, and G. Gabbiani, manuscript submitted for publication). In all cases, only the α -spot was recognized by anti- α sm-1 (Fig. 2, *b* and *e*). We controlled that actin isoforms did transfer

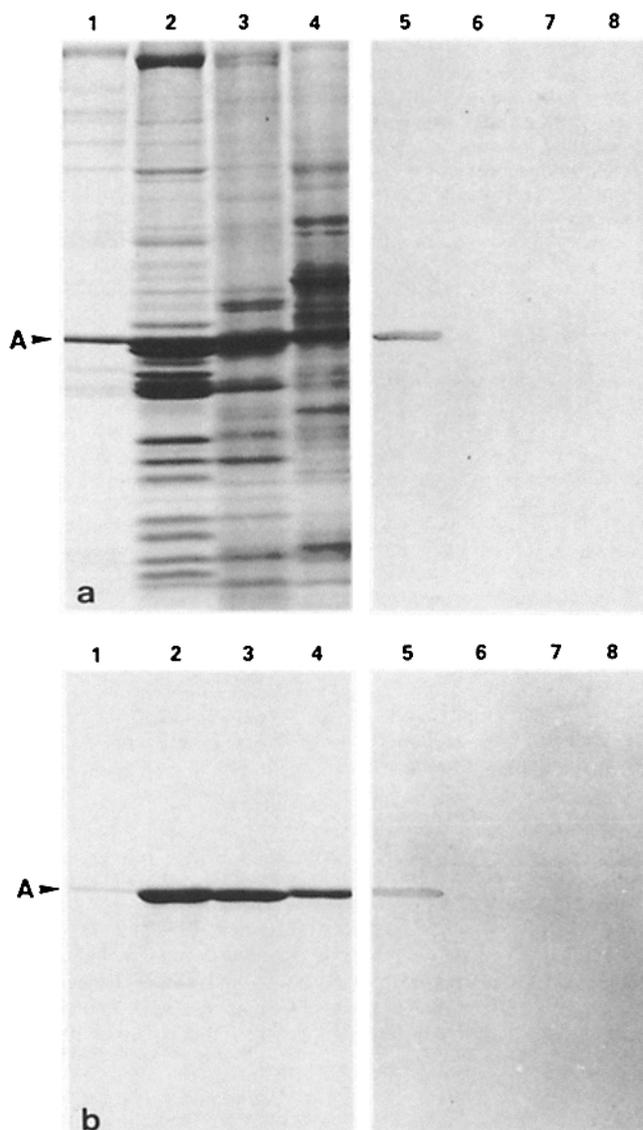


Figure 1. Immunoblotting with anti- α sm-1 of different tissue or cell total extracts (*a*) and of purified actins (*b*). (*a*) Coomassie Blue-stained 5–20% gradient SDS PAGE of rat aorta (lane 1), rat striated muscle (lane 2), rat myocardium (lane 3), and human fibroblasts (lane 4). Nitrocellulose sheet corresponding to SDS PAGE after incubation with anti- α sm-1 shows a positive reaction only with rat aorta actin (lane 5). (*b*) Coomassie Blue-stained 5–20% gradient SDS PAGE of purified actins from bovine aorta (lane 1), chicken gizzard (lane 2), human platelets (lane 3), and rabbit striated muscle (lane 4), loaded in ~ 10 -fold excess compared to bovine aortic actin. A positive reaction is seen only with bovine aortic actin. A, actin.

equally well on the nitrocellulose filters by staining them with a rabbit anti-actin recognizing all isoforms (Fig. 2, *c* and *f*). Taken together, these results indicate that anti- α sm-1 reacts only with the α -smooth muscle isoform of actin within a broad range of concentrations and in the presence of an excess of other actin isoforms.

The specificity of anti- α sm-1 was also tested by means of immunofluorescence on various tissues and cells (Fig. 3). As previously said, immunofluorescence on frozen sections of chicken gizzard with anti- α sm-1 resulted in a strong positive

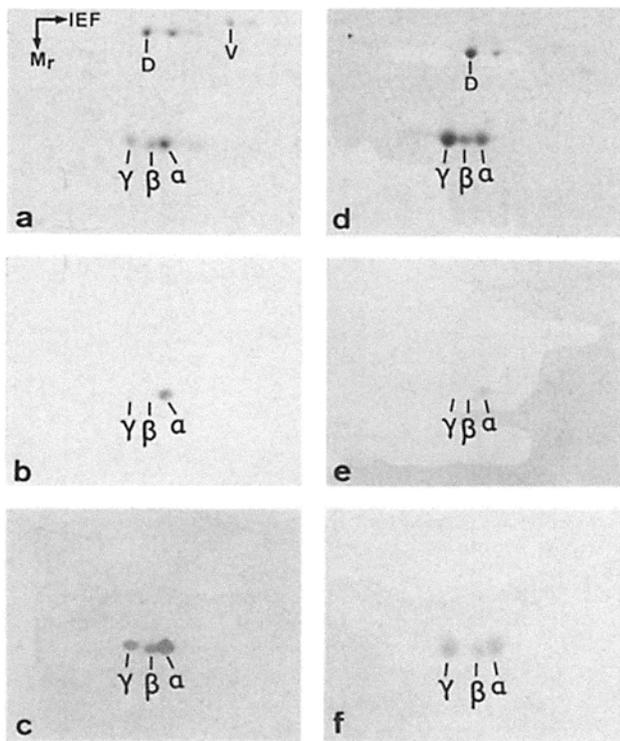


Figure 2. Immunoblotting with anti- α -sm-1 of human myometrium and gastric muscle total extracts. (a and d) Coomassie Blue-stained 2D-PAGE of human myometrium (a) and gastric muscle (d) shows the separation of actin isoforms in three spots (γ -smooth muscle and γ -cytoplasmic actins co-migrate). (b and e) After incubation of the nitrocellulose paper with anti- α -sm-1, a single peroxidase positive spot is seen. (c and f) When incubation of the nitrocellulose paper is made with a rabbit antibody recognizing all actin isoforms, peroxidase activity is distributed in the three spots corresponding to those of the gel (a and d), showing that actin isoforms transfer equally well from the gel to the nitrocellulose paper. D, desmin; V, vimentin.

stain of blood vessels but parenchymal SMCs remained negative within an IgG concentration range of 50 μ g/ml to 0.1 μ g/ml (Fig. 3 a). In rat intestine, SMCs of the outer longitudinal and inner circular layers and of the muscularis mucosae were stained by anti- α -sm-1; epithelial cells, including brush borders, were not stained. Fibroblasts in rat and human dermis, as well as striated muscle and myocardial cells (Fig. 3 c), were not stained by anti- α -sm-1. Anti- α -sm-1 decorated stress fibers in cultured rat vascular SMCs and in a cell line containing α -smooth muscle actin, BC3H1 (50). In human embryo lung cells, a fibroblastic cell line, staining was always negative. However, in rat dermal fibroblast primary cultures and, to a lesser extent, at the fourth passage, some cells showed stress fibers positive for α -smooth muscle actin, suggesting that primary and passaged rat dermis fibroblasts contain a contaminant population of SMCs (Fig. 3 e). In passaged cultures of chick embryo fibroblasts, stress fibers positive to anti- α -sm-1 were seen in a high proportion of cells, suggesting that this culture consists mainly of SMCs.

Studies on Arterial SMCs

Staining of rat or human aorta showed a positive reaction in

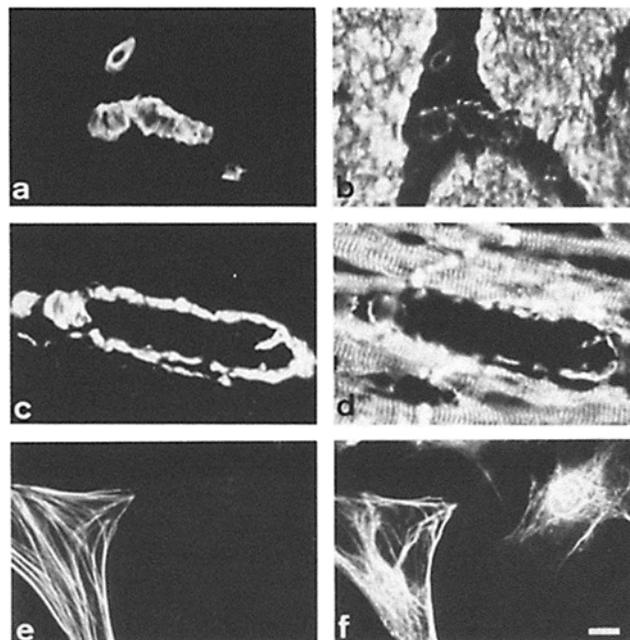


Figure 3. Double immunofluorescent staining with anti- α -sm-1 (a, c, and e) and anti-desmin (b and d) or anti-vimentin (f) on chicken gizzard (a and b), rat myocardium (c and d) and passaged rat dermal fibroblasts (e and f). Anti- α -sm-1 stains only blood vessels on chicken gizzard (a) and rat myocardium (c), whereas chicken gizzard parenchymal cells and cardiocytes are negative; anti-desmin antibodies stain blood vessels as well as chicken gizzard parenchymal cells (b) and cardiocytes (d). Note that in the chicken gizzard blood vessel more cells are stained with anti- α -sm-1 than with anti-desmin (a and b). In passaged rat dermal fibroblasts, some cells show stress fibers stained with anti- α -sm-1 whereas others are negative for this antibody (e); all cells are positive for anti-vimentin which stains typically the intermediate filament network (f). Bar, 10 μ m.

the media and in certain vasa vasorum but not in adventitia or endothelium (Fig. 4). Double immunofluorescent staining of rat and human aortic media with anti-vimentin and anti- α -sm-1 showed that both arteries contain an SMC population expressing vimentin, but not α -smooth muscle actin (Fig. 4). Cytospins of enzymatically digested aortic media of 6-wk-old adult rats showed that this cell population constitutes $8 \pm 5\%$ of aortic media SMCs. Isolated SMCs negative for α -smooth muscle actin were also negative for Factor VIII and desmin. The proportion of α -smooth muscle actin-negative SMCs varies during development: in 5-d-old rats, as much as $49 \pm 3\%$ of the cells of the aortic media are negative for α -smooth muscle actin, but positive for vimentin (Fig. 5, a and b). Thus, differentiation of aortic SMCs takes place in the rat after birth as far as α -smooth muscle actin is concerned. When adult rat aortic SMCs are placed in culture in the presence of 10% FCS (46), the proportions of anti- α -sm-1 strongly stained (more than four positive stress fibers), weakly stained (between one and four positive stress fibers), and negative cells were as follows: $68 \pm 10\%$, $14 \pm 8\%$, and $17 \pm 3\%$, respectively, 5 d after plating, and then $26 \pm 3\%$, $48 \pm 1\%$, and $26 \pm 4\%$, respectively, at the fifth passage (Fig. 5, c and d). This suggests that SMC growth in the presence of 10% FCS is characterized by a gradual decrease of α -smooth muscle actin in cells derived from replicating

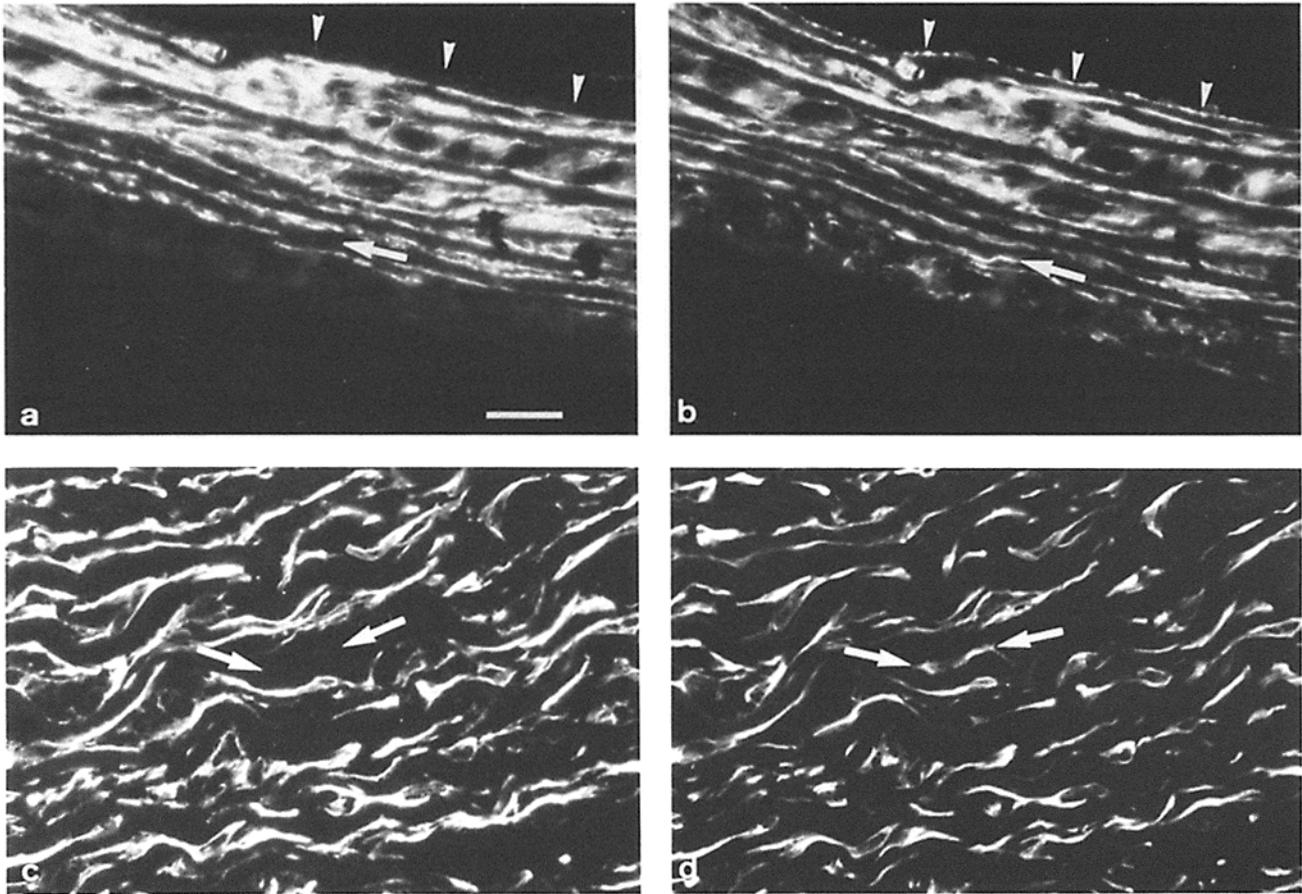


Figure 4. Double immunofluorescence with anti- α -sm-1 (*a* and *c*) and anti-vimentin (*b* and *d*) on rat (*a* and *b*) and human (*c* and *d*) aorta. In the rat aorta, endothelial cells (*arrowheads*) and adventitial fibroblasts are stained with anti-vimentin (*b*) but not with anti- α -sm-1 (*a*). Anti- α -sm-1 and anti-vimentin stain medial SMCs of the rat and human media. However, few rat and human medial SMCs are stained with vimentin but not with anti- α -sm-1 (*arrows*). Note that the staining pattern of anti- α -sm-1 does not always fully correspond to that of anti-vimentin in cells which are stained by both antibodies. Bar, 10 μ m.

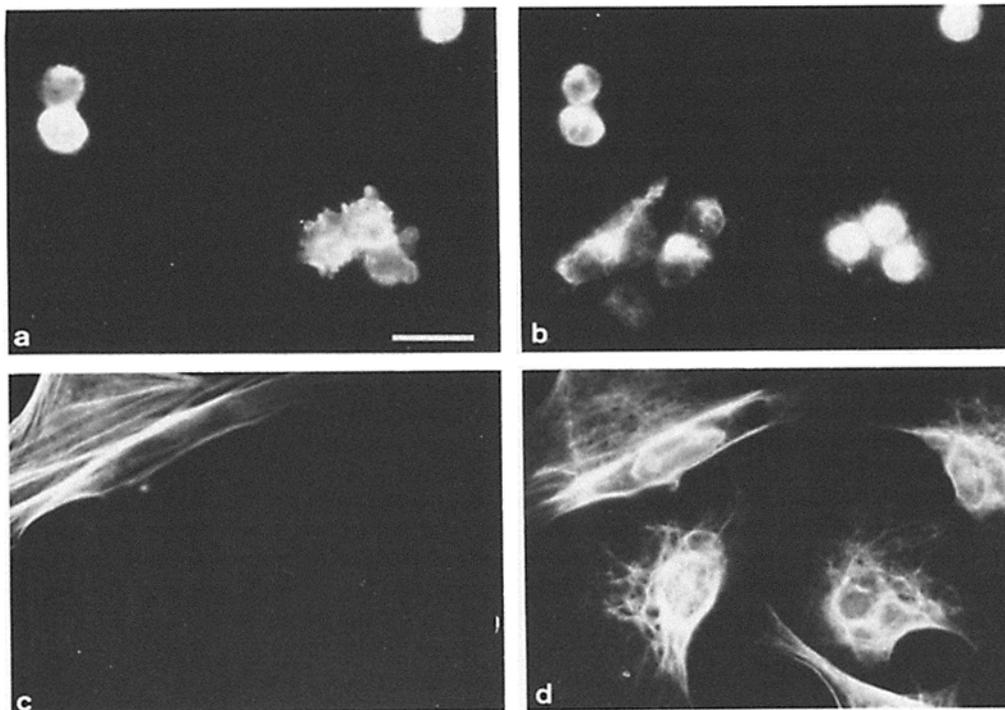


Figure 5. Double immunofluorescence with anti- α -sm-1 (*a* and *c*) and anti-vimentin (*b* and *d*) on cytopsin of 5-d-old rat aortic medial SMCs (*a* and *b*) and nonconfluent primary culture of adult rat aortic medial SMCs (*c* and *d*). Both conditions demonstrate vimentin-positive, anti- α -sm-1-negative cells together with vimentin-positive, anti- α -sm-1-positive SMC. Stress fibers are clearly seen in the anti- α -sm-1-positive SMCs (*c*). Bar, 10 μ m.

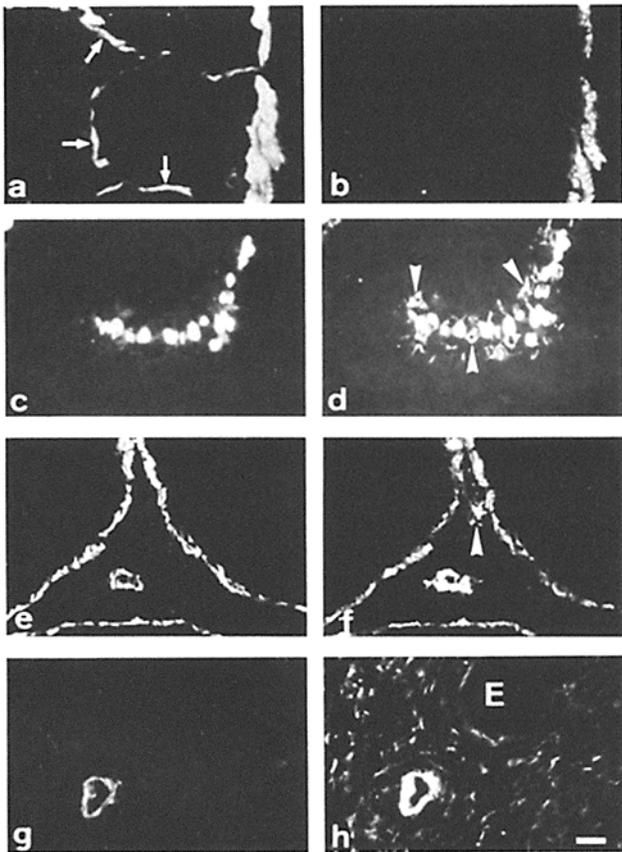


Figure 6. Double immunofluorescent staining with anti- α -sm-1 (a, c, e, and g) and anti-desmin (b, d, f, and h) on rat intestine (a-d), rat testis (e and f), and rat uterus (g and h). In a and b, intestinal villi are cut transversally, while in c and d they are cut longitudinally. SMCs stained by anti-desmin and anti- α -sm-1 are visible in the intestinal muscularis (a) and muscularis mucosae (a and c) and in small blood vessels of the testis interstitium (e and f) and uterine stroma (g and h). The myoid cells surrounding seminiferous tubules are also positive for anti- α -sm-1 (e) and anti-desmin (f). Cells positive for anti- α -sm-1 and negative for anti-desmin are visible in the intestinal muscularis mucosae (arrows) (a). Cells positive for anti-desmin but not for anti- α -sm-1 (arrowheads) are visible in the muscularis mucosae (d), testis interstitium (f), and uterine stroma (h). E, epithelium. Bar, 10 μ m.

α -smooth muscle actin-positive SMC and only by a relatively low level of replication of α -smooth muscle actin-negative cells.

We investigated further whether α -smooth muscle actin-negative cells have a greater replicative activity than other aortic medial SMCs when placed in culture in the presence of 10% FCS. SMCs entering for the first time in S-G2 phases were sorted by cytofluorimetry according to their DNA content. After double immunofluorescent staining, the percentage of cells positive for vimentin but not for α -smooth muscle actin was the same for cells in G1 and for those in S-G2 phase of the cell cycle, thus confirming that 10% FCS does not stimulate selectively α -smooth muscle-negative SMCs.

Comparison of Desmin and α -Smooth Muscle Actin Distribution in Cells of Different Tissues

Desmin is a widely accepted general marker of myogenic dif-

ferentiation (33, 40); however, it is known that some vascular SMCs do not contain desmin (12, 42, 53). Thus, the presence of α -smooth muscle actin rather than the presence of desmin may represent a specific marker of SMCs (Skalli, O., J. Vandekerckhove, and G. Gabbiani, manuscript submitted for publication). We have verified in various normal tissues the distribution of α -smooth muscle actin and desmin by double immunofluorescent staining. In the arterial wall of rat and man, desmin-positive cells appear always α -smooth muscle actin-positive, but the reverse is not true. When expressed as percentage of total cells, α -smooth muscle actin-positive and desmin-negative cells are $35 \pm 4\%$ in 5-d-old rats and $27 \pm 6\%$ in 6-wk-old rats.

Cells positive for α -smooth muscle actin and negative for desmin are not found only in the aortic media, but also focally in the muscularis mucosae of the rat intestine (Fig. 6, a and b) and around hair follicles of the rat dermis. Finally and surprisingly, cells containing desmin but not α -smooth muscle actin were found in rat intestinal submucosa (Fig. 6, c and d), testis interstitium (Fig. 6, e and f) and uterine stroma (Fig. 6, g and h) (observed in collaboration with Mr. T. Soldati, Department of Pathology, University of Geneva, Geneva, Switzerland). The desmin-positive, α -actin-negative cells of the testis are distinct from the classical myoid cells surrounding seminiferous tubules (4) which were positive for both anti-desmin and anti- α -sm-1 (Fig. 6, e and f).

Distribution of α -Smooth Muscle Actin in Normal Mammary or Salivary Glands and in Breast Carcinomas

Myoepithelial cells have been shown to express cytokeratin (8) and smooth muscle myosin (29). After immunofluorescent staining of human mammary gland and rat submaxillary gland with anti- α -sm-1, myoepithelial cells surrounding the acini and ducts were clearly positive (Fig. 7, a and c). The staining of myoepithelial cells was superimposable to that obtained with anti-smooth muscle myosin; in addition, as previously described (8), myoepithelial cells were decorated by prekeratin antibodies. Epithelial and stromal cells of the normal mammary and salivary glands were negative after immunofluorescent staining with anti- α -sm-1.

The presence of α -actin in the rat submaxillary gland was further tested by immunoblots after 2D-PAGE (Fig. 8). Only the α -spot was recognized by anti- α -sm-1. A similar result was found for ductal carcinoma of the breast (data not shown).

Infiltrating ductal carcinoma of the breast is characterized by nests of tumor cells scattered in a dense, fibrous stroma. Stromal cells have been described as myofibroblasts (43, 54; for other references see 45). Double immunofluorescence with anti-prekeratin and anti- α -sm-1 showed that, as expected, tumor cells and the few myoepithelial cells still present in tumor islets were stained with anti-prekeratin. Anti- α -sm-1 positively stained myoepithelial cells (Fig. 7 e); moreover, surprisingly, stromal cells were also positive after anti- α -sm-1 staining (Fig. 7 e), unlike stromal cells of the normal breast and typical myofibroblasts of human or experimental wound granulation tissue (data not shown). Thus, in some fibrotic reactions, the main cellular components appear to be SMCs.

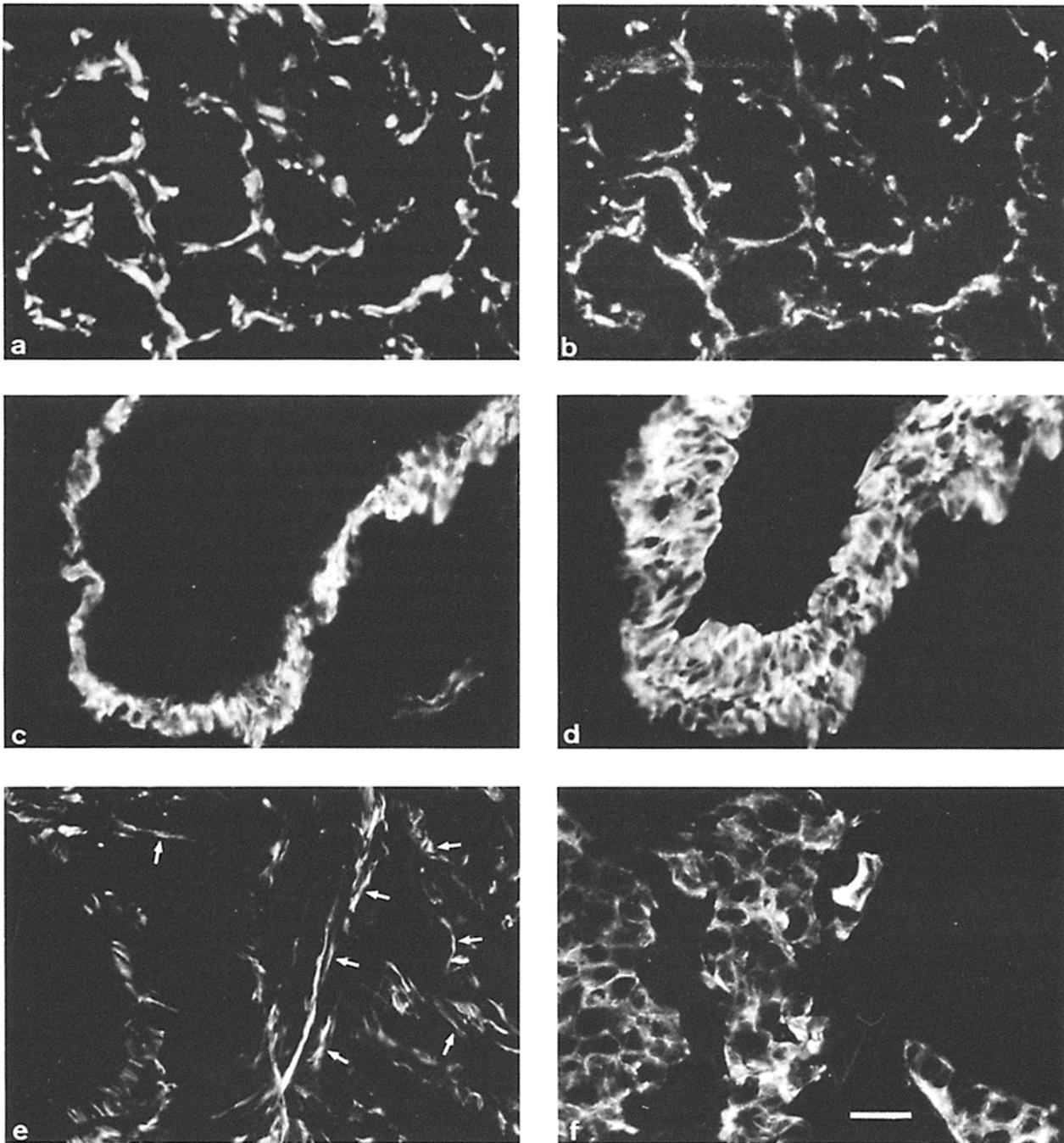


Figure 7. Double immunofluorescent staining for α -smooth muscle actin (*a*, *c*, and *e*), smooth muscle myosin (*b*), and prekeratin (*d* and *f*) on rat submaxillary gland (*a* and *b*), human resting mammary gland (*c* and *d*), and infiltrating duct carcinoma (*e* and *f*). In the rat submaxillary gland, myoepithelial cells staining with anti- α sm-1 (*a*) and anti-aortic smooth muscle myosin (*b*) are superimposable; acini are not stained with both antibodies. In the human resting mammary gland, myoepithelial cells brightly stained with anti- α sm-1 (*c*) are located around the prekeratin-positive, anti- α sm-1-negative ductal epithelium (*d*); no connective tissue cells are stained with either antibody (*c* and *d*) with the exception of a small blood vessel stained with anti- α sm-1 (*c*). In the infiltrating duct carcinoma, some myoepithelial cells positive for α -smooth muscle actin and prekeratin are present within the typical keratin-positive, anti- α sm-1-negative, infiltrating tumoral cells (*e* and *f*). Moreover, numerous cells of the stroma are positive to anti- α sm-1 (arrows) (*e*). Bar, 10 μ m.

Discussion

Production of polyclonal or monoclonal antibodies specific for a given actin isoform has been attempted using the following various approaches: use as immunogen of total actin from chicken (16) or lower species (30, 44) and of an NH₂-terminal peptide (5, 34); or serial affinity purifications of

polyclonal antibodies (17, 36, 38). All these methods have resulted in the production of antibodies directed against at least two isoforms with the possible exception of Roustan et al. (38) who purified an antibody subpopulation specific for α -sarcomeric actin from a serum of a rabbit sensitized with skeletal muscle actin. Some authors have not characterized the actin isoform(s) recognized by their antibodies obtained

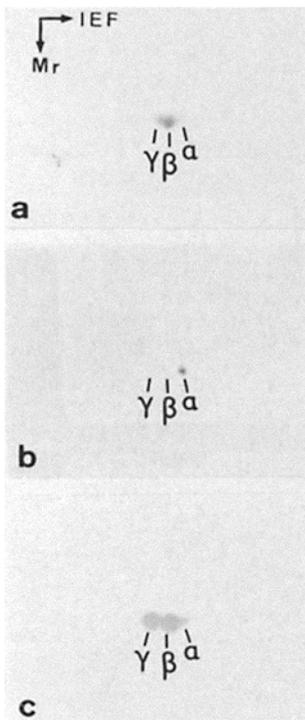


Figure 8. Immunoblotting with anti- α sm-1 of rat submaxillary gland total extract. (a) Coomassie Blue-stained 2D-PAGE of rat submaxillary gland shows that this tissue contains a predominance of β -actin but also α - and γ -actins. (b) After incubation of the nitrocellulose paper with anti- α sm-1, a single, small peroxidase-positive spot is seen. When incubation of the nitrocellulose paper is made with a rabbit antibody recognizing all actin isoforms, peroxidase activity is distributed in the three spots corresponding to those of the gel (a), showing that the three actin isoforms were transferred from the gel to the nitrocellulose paper.

after immunization with actin and selectively decorating SMCs (17).

We have produced a monoclonal antibody against the acetylated NH_2 -terminal decapeptide of α -smooth muscle actin which reacts specifically with α -smooth muscle actin when tested by means of immunoblotting, ELISA, and immunofluorescence. To our knowledge, the acetylation of the NH_2 -terminal residue is the only posttranslational modification which has been described in this location (57). However, we cannot exclude that other possible posttranslational modifications of the NH_2 -terminal peptide are not recognized by anti- α sm-1. In any event, we have not noted discrepancies between the distribution of anti- α sm-1 immunofluorescent staining and the presently known distribution of α -actin by biochemical analysis.

Raising polyclonal antibodies against a short peptide bearing amino acid sequences peculiar to a given protein isoform is a recognized method to produce antibodies against this isoform. However, this method appears insufficient as far as actin isoforms are concerned both from previous work (5, 34) and from our present experience with the immunization of rabbits by means of the NH_2 -terminal decapeptide conjugate of α -smooth muscle actin (see Results). Thus, production of monoclonal antibodies from mice hyperimmunized with NH_2 -terminal α -smooth muscle decapeptide conjugate appears the most valid approach in order to achieve specificity for α -smooth muscle actin and possibly other actin isoforms. However, even with this procedure, about half of the positive hybridoma supernatants were not specific for α -smooth muscle actin, indicating that immunization of mice with the NH_2 -terminal decapeptide conjugate of α -smooth muscle actin resulted in at least two antibody populations: one specific for α -smooth muscle actin and the other cross-reacting with other actin isoforms. This suggests that the NH_2 -terminal decapeptide of α -smooth muscle actin bears

at least two epitopes: one shared by different actin isoforms, and the other specific for α -smooth muscle actin. We do not know which portion of the NH_2 -terminal decapeptide of α -smooth muscle actin constitutes the epitope recognized by anti- α sm-1. One may speculate that this epitope contains the sequence Glu-Asp-Ser-Thr which differs from all the other actin isoforms. However, precise determination of this important point requires further work such as that reported by Wehland et al. for an α -tubulin monoclonal antibody (58).

Several studies (47, 56, 57; Skalli, O., J. Vandekerckhove, and G. Gabbiani, manuscript submitted for publication) have indicated that in mammals, SMCs containing tissues are characterized by the constant presence of α -, β -, and γ -actin isoforms in various proportions while fibroblastic tissues contain only β - and γ -cytoplasmic isoforms. Anti- α sm-1 may represent a good marker of smooth muscle differentiation at the cellular level as indicated by the results of immunofluorescent staining of rat dermal "fibroblasts" or chicken embryo "fibroblasts." In view of our results (this work; Skalli, O., J. Vandekerckhove, and G. Gabbiani, manuscript submitted for publication), one should consider the possibility of revising the term "fibroblastic" for cell lines containing α -smooth muscle actin (see for instance 27, 60).

Anti- α sm-1 stained blood vessels in rat striated muscle and in chicken gizzard; however, 2D-PAGE (see references 24, 39 for chicken gizzard), analysis of the NH_2 -terminal peptides of actin (56, 57), and immunoblotting (our results) each failed to detect any α -smooth muscle actin in these tissues. Additional experiments (not reported here) using rat aortic SMCs co-cultured with a human embryo lung fibroblast line have shown that when the cell preparations have <5% α -smooth muscle actin-containing cells, a Coomassie Blue-stained 2D-PAGE showed consistently no α -actin spot. All these results indicate that anti- α sm-1 could be useful in the study of SMC development at the cellular level and in the identification of SMC tumors in surgical pathology specimens. Moreover, anti- α sm-1 could be used in order to affinity purify α -smooth muscle actin.

Immunofluorescent staining with anti- α sm-1 has shown that arterial SMCs in vivo and in vitro consist of three populations as far as their cytoskeletal features are concerned. One is vimentin, desmin, and α -smooth muscle actin positive; the second is vimentin and α -smooth muscle actin positive, but desmin negative; and the third is vimentin positive, but α -smooth muscle actin and desmin negative. We cannot exclude that this last cell population contains γ -smooth muscle actin; however, our previous work on cytoskeletal features of aortic SMCs during development (23) and the present results after staining aortic SMCs of newborn and adult animals suggest that the arterial media of young, and to a lesser extent, adult rats and men contain a cellular population lacking the cytoskeletal markers of muscular differentiation. The role of this population in repair and pathology of the vascular wall is not known.

Previous studies have shown that replication of SMCs in vivo and in vitro is characterized by a decrease of their α -smooth muscle actin content (35, 46). The observation that SMCs in culture show a decrease of anti- α sm-1 staining intensity rather than an important increase of anti- α sm-1-negative cells suggests that the decrease of α -smooth muscle actin content in replicating SMCs is due to a diminished α -smooth muscle actin per cell rather than to a selection of

an α -smooth muscle actin-negative population. This is supported by the finding that after plating, anti- α -sm-1 negative SMCs have the same replicative activity as anti- α -sm-1-positive SMCs. In any event, it appears that these α -smooth muscle actin- and desmin-negative SMCs are not more stimulated to replicate in primary culture than other SMCs by the growth factors present in FCS.

Cultured rat aortic SMCs have been studied by means of an actin antibody selected after fusion of myeloma cells with lymphocytes from a mouse immunized with chicken gizzard actin and reported to react with α - and γ -smooth muscle actins (16, 35). In passaged cultures, unlike anti- α -sm-1, this antibody did not stain SMCs (16). We have no explanation for the discrepancy between these results and the present findings. However, the constant presence in cultured SMCs of low but significant amounts of α -smooth muscle actin, of α -smooth muscle actin synthesis, and α -smooth muscle actin mRNA has been shown by several biochemical techniques (1, 46). The same has been reported in SMC lines (9, 50). Our results after staining with anti- α -sm-1 confirm at the cellular level these previous analyses.

The staining of myoepithelial cells with anti- α -sm-1 raises the question of the origin of these peculiar cells. On the basis of staining with prekeratin antibodies, they have been proposed to be of epithelial origin in accordance to embryological data (8, 18). Whatever the answer, it appears that myoepithelial cells contain both epithelial and mesenchymal differentiation markers. Thus, cytoskeletal features, although powerful indicators, may not always represent absolute markers of cellular origin.

Staining with anti- α -sm-1 may help in the characterization of stromal cell heterogeneity in various organs. Thus, we know now that α -smooth muscle actin-positive, desmin-negative SMCs are present in the intestinal muscularis mucosae and around hair follicles, in addition to those well characterized in the vascular wall (12, 42, 53). Moreover, a desmin-positive, α -smooth muscle actin-negative population of stromal cells has been identified in the stroma of intestine, testis, and uterus. We cannot exclude that this cell population contains γ -smooth muscle actin or that the NH₂-terminal decapeptide of α -smooth muscle actin is occluded and thus cannot be recognized by anti- α -sm-1. This is a general restriction to the use of any monoclonal antibody, but so far when α -actin was found by biochemical means, it was also detected by immunofluorescence with anti- α -sm-1. In any event, the functional significance of these desmin-positive, α -actin-negative cells is presently unknown.

Finally, the finding that stromal cells of mammary carcinoma contain α -smooth muscle actin may throw some new light on the pathophysiological mechanisms of fibrotic reactions. The observation that during wound healing and fibrocontractive diseases fibroblast modulate into a cell type possessing contractile features, called the myofibroblast (10; for review see 45), has allowed a better understanding of the mechanisms of connective tissue retraction. The present finding, that during a well-established fibrotic stromal reaction the cells involved show smooth muscle properties, may define a new category of fibrotic reactions among fibromatoses, a complex group of pathological changes showing features of inflammatory and tumoral lesions (43, 45, 54). It has been reported that mammary carcinoma cells produce one or more factors stimulating the replication of stromal fibro-

blasts (15). Our findings suggest that this or these factor(s) are also capable of stimulating smooth muscle differentiation.

In conclusion, anti- α -sm-1 appears to represent a useful tool for the study of smooth muscle differentiation and characterization in normal and pathological conditions.

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